Determining Drug Sensitivity

Use of the Gel Diffusion Method

VAN VLECK CHAMBERS, *M.D.*, Palo Alto

• **A study was carried out to determine whether the double diffusion gel test when applied to the serum of patients with clear-cut penicillin reactions of various types, might be useful for demonstrating the presence of precipitating antibody. Results did not demonstrate the antibody.**

The difference in results with this test obtained by various workers was not explained by the observations in this study.

Other approaches to determination of the mechanism of the penicillin reaction are discussed, and it is noted that the hemagglutination test, newly applied to the penicillin reaction problem, may be useful after further investigation.

ATTEMPTS TO DEMONSTRATE the presence in human serum of precipitating antibody against drugs that produce sensitivity reactions have resulted in conflicting claims. The original adaptation of the gel diffusion test for precipitins was made in 1957 by Muelling and co-workers[24,25] who used an agar-stabilized tube technique. The serum of patients thought to have had a drug reaction was tested and a large number of positive tests were reported. We have used the double diffusion method of Ouchterlony,[28] which is similar in principle but somewhat different in detail. Ouchterlony's technique has been used to demonstrate human serum precipitating antibody in a variety of conditions, including histoplasmosis[14] and thyroiditis,[8] and to differentiate antibodies against such antigens as streptolysin-O,[11] stinging insects,[41] tubercle bacillus protein,[32] trichinella,[46] amaranth-chenopod pollen,[47] house dust[45] and many others.[48]

Drug reactions are becoming more common as more drugs are made available for routine use. Penicillin probably causes 80 per cent of all drug reactions.[15] There is no thoroughly satisfactory test for recognizing penicillin reactions.[12,35,38,39] Skin tests for penicillin sensitivity, whether scratch or intradermal, have caused death and severe anaphylaxis.[21,34] They often result in false positive and false negative reactions,* and there is considerable doubt whether skin tests predict or diagnose accurately,[18,34] although they may be of some help if positive.[9,10,20] Attempts have been made to incubate penicillin with gamma globulin[36] and sulfonamides with gamma globulin[16] and to use these mixtures as complete antigens in skin testing for hypersensitivity to these drugs. There are no reports of the further success of these methods,[18] and at least one earlier reported failure.[9]

Thus, the available methods for distinguishing drug sensitivity are unreliable, misleading and dangerous. A reliable method of demonstrating drug sensitivity in humans would be useful for predicting hypersensitivity, for definitive diagnosis in patients suspected of having had previous reactions, and for distinguishing drug reactions from other diseases. If results with the gel diffusion technique such as those reported by Muelling could be duplicated, an extremely valuable tool would be available for both clinical and research use.

Presented before the Section on Allergy at the 89th Annual Session of the California Medical Association, Los Angeles, February 21 to 24, 1960.

From the Department of Pediatrics, Section of Pediatric Allergy, Palo Alto Medical Clinic, and Division of Immunology and Allergy, Palo Alto Medical Research Foundation.

*References 2, 4, 5, 9, 27, 33, 37, 40, 44.

METHOD

Patients were selected from the Palo Alto Medical Clinic if they had reacted to penicillin or other drugs. Penicillin reactors were sought in particular, however, because they are relatively common,[2,27] technique and materials could be standardized if only one drug were considered, and reactions had been documented in the patient's charts in most instances. My own observation or clearly described and recorded observation by another physician was the source of validation of the presence and type of reaction in 68 per cent of the cases studied. The remaining 32 per cent of the patients were included only after interview confirmed the details of their reactions. All patients were personally interviewed for all other information included in this report.

The gel diffusion technique was introduced by Oudin[29] and amplified as the double diffusion plate by Ouchterlony.[28] The method employed in this study is the double diffusion plate as used by the Department of Immunology and Allergy at the Palo Alto Medical Research Foundation.

The agar plates are prepared as follows:

Materials:	Difco Bacto-Agar®	10.00 gm.
	NaCl	4.25 gm.
	Monobasic potassium phosphate	0.19 gm.
	Distilled water	450.00 cc.

TABLE 1.—*Age and Sex Characteristics of Patients with Reactions*

Age Groups in Years	Per Cent Total Reactors		Total No. Reactors		Reactions to Penicillin Administered: Orally Only		By Injection Only		Orally and Injected Concomitantly		Reactions to Other Drugs	
	M	F	M	F	M	F	M	F	M	F	M	F
0 to 10	0	5	0	1	0	0	0	0	0	1	0	0
11 to 20	5	5	1	1	0	1	1	0	0	0	0	0
21 to 30	5	22	1	5	0	1	1	4	0	0	0	0
31 to 40	9	18	2	4	1	0	0	2	1	0	0	2
41 to 50	13	0	3	0	0	0	3	0	0	0	0	0
51 to 60	9	9	2	2	0	0	2	2	0	0	0	0
Total	41	59	9	13	1	2	7	8	1	1	0	2

TABLE 2.—*Allergic Characteristics of Patients with Reactions*

History of	Total Per Cent	Total No. Patients	Reactions to Penicillin Administered: Orally Only	By Injection Only	Orally and Injected Concomitantly	Reactions to Other Drugs
Personal allergy	22	5	1	3	1	0
Family history allergy	35	8	1	5	0	2
Personal or family allergy	45	10	2	6	1	1
Previous exposure to drug causing reaction	68	15	3	11	1	0
Other drug allergy	8	2	1	0	0	1
Poliomyelitis vaccine reaction	0	0	0	0	0	0
Insect bite anaphylaxis	0	0	0	0	0	0

Salts and agar are added to boiling water and stirred until melted. The mixture is then placed in tubes in 27 cc. aliquots, autoclaved, and capped for storage. When ready to use, the mixture is melted in a boiling water bath. A 9 cm. sterile plastic Petri dish is readied, into which is first poured 3.0 cc. of 1:1000 aqueous Merthiolate. The melted agar is then carefully added and swirled slowly, then allowed to jell. Wells are cut in the agar with the end of a glass tube approximately 0.8 mm. in diameter. A cluster of wells is made by placing four wells at equal intervals around the periphery of a fifth central well, with the inner edges of the peripheral wells each 5 mm. from the outer edge of the central well. Four such clusters are placed in one 9 cm. Petri dish.

Varying dilutions of serum are put into each central well of each cluster. In this study we used undiluted serum and serum diluted 1:10, 1:50 and 1:100 with normal saline solution. The peripheral wells contained a solution of the antigen (drug) in various dilutions. Drug concentrates were made as follows: Sodium penicillin 500,000 units per cubic centimeter; procaine penicillin 500,000 units per cubic centimeter; potassium penicillin 330,000 units per cubic centimeter; benzathine penicillin 1.2 million units per cubic centimeter; tetanus antitoxin 1,500 units per cubic centimeter; and chloramphenicol 400 mgm. per cubic centimeter. Each concentrate was used as such and, in addition, diluted 1:10, 1:50, 1:100, 1:500, 1:1000, 1:5000, 1:10,000, 1:50,000 and 1:100,000 in saline solution. Thus, each serum concentration was exposed to each of the ten penicillin concentrations. Not all the penicillin-sensitive patients were tested against all the penicillin preparations noted here, but each was tested against the type thought to have been the cause of the reaction, and usually to either the sodium or potassium aqueous forms as well.

The completed plates were kept at room temperature in completely dark, high-humidity containers and read at 24 hours, 48 hours, and 72 hours. All were last read at seven days, 60 per cent were last read at 14 days, and 40 per cent were last read at 21 days.

Tests were made of patients with reactions to oral penicillin alone (3 patients), to injected penicillin alone (15 patients), to oral and injected penicillin concomitantly (2 patients), to tetanus antitoxin (1 patient), and to chloramphenicol (1 patient). Also tested were two patients who had had penicillin but had not had a reaction, and three patients who were currently receiving penicillin with no reaction.

RESULTS

Some general characteristics of the series of patients studied are recorded in Tables 1 and 2. These characteristics resemble certain of those noted in other studies of drug reactions,* suggesting that this is a valid sample even though some of the occurrences within the sample are too few to be significant. Percentages must be interpreted in relation to the total number of occurrences.

*References 5, 10, 18, 21, 27, 34.

TABLE 3.—*Data Characteristics of Patients with Reactions*

Source	Per Cent Total Reactors	Total No. Reactors	Reactions to Penicillin Administered: Orally Only	By Injection Only	Orally and Injected Concomitantly	Reactions to Other Drugs
Source of information*:						
Personal observation	14	3	0	1	0	2
Clinic chart	54	12	2	8	2	0
Patient's history	32	7	1	6	0	0
Total	100	22	3	15	2	2
Time interval since reaction occurred (in years):						
Years ago:						
0 to 1	40	9	2	4	1	2
1 to 2	18	4	1	2	1	0
2 to 3	5	1	0	1	0	0
3 to 5	14	3	0	3	0	0
5 to 8	18	4	0	4	0	0
8 to 12	5	1	0	1	0	0
Total	100	22	3	15	2	2

*Regarding reaction only (other information by personal interview).

TABLE 4.—*Reaction Characteristics of Patients with Reactions*

Symptoms	Per Cent Total Reactors	Total No. Reactors	Reactions to Penicillin Administered: Orally Only	By Injection Only	Orally and Injected Concomitantly	Reactions to Other Drugs
Type of symptoms:						
Angio-edema, urticaria	77	17	2	12	2	1
Shock	18	4	1	2	0	1
Rash	5	1	0	1	0	0
Total	100	22	3	15	2	2
Severity of symptoms:						
Slight	14	3	0	2	1	0
Moderate	63	14	2	10	1	1
Severe	23	5	1	3	0	1
Total	100	22	3	15	2	2

A total of 27 patients was tested of whom 22 had had clear-cut reaction to drugs, and 5 had had no reaction to any drug. There were 41 per cent males and 59 per cent females, and their ages ranged from 4 to 60 years, with 54 per cent of the patients between 21 and 40 years of age (Table 1). The largest single category consisted of 15 patients who reacted to penicillin given by injection.

Twenty-two per cent of patients had a personal and 35 per cent a family history of major allergic disease (Table 2); and the combined total of those who had either or both made up 45 per cent of the series. There were few reactions to drugs other than those which caused the reactions studied. Twenty-one who had penicillin reactions had had poliomyelitis vaccine within two years of the time of the test, and none had had a reaction. There were no cases of insect bite anaphylaxis.

No previous exposure to the drug causing the reaction was known in 32 per cent of the patients. This lack of history of exposure may have been the result of the patient's failure to remember correctly or of incomplete clinical records. Also, for those who had reactions of the serum sickness type, a history of previous exposure was not expected.

Further characteristics of the patients with reactions are shown in Tables 3, 4 and 5. Forty per cent of the patients had had reactions within the 12 months preceding the test (Table 3). Seventy-seven per cent of the reactions were urticaria or angio-edema or both, and 18 per cent were severe shock reactions (Table 4). The reactions were moderate to severe in 63 per cent of the cases. Most patients had had one to six doses of the responsible drug before reaction occurred, and over 50 per cent had symptoms within two days of the last exposure (Table 5).

Table 6 shows the characteristics of the test results. All tests were negative. A total of 50 tests were performed, and almost half of all patients had tests performed for more than one type of penicillin.

DISCUSSION

Failure to demonstrate precipitating antibody in persons with a sensitivity reaction to drugs was not

TABLE 5.—*Reaction Characteristics of Patients with Reactions*

Doses	Per Cent Total Reactors	Total No. Reactors	Reactions to Penicillin Administered: Orally Only	By Injection Only	Orally and Injected Concomitantly	Reactions to Other Drugs
Number of doses of drug before reaction:						
1 to 2	68	15	1	12	0	2
3 to 6	24	5	2	1	2	0
7 to 10	8	2	0	2	0	0
Total	100	22	3	15	2	2
Number of days after last dose that reaction began:						
1 to 24 hours	36	8	1	5	0	2
1 to 2	23	5	1	4	0	0
3 to 4	9	2	1	1	0	0
5 to 8	14	3	0	1	2	0
9 to 12	18	4	0	4	0	0
Total	100	22	3	15	2	2

TABLE 6.—*Test Characteristics of Reactors (22) and Nonreactors (5)*

Drug Tested	Per Cent Total Patients Tested	Total No. Patients Tested	Reactions to Penicillin Administered: Orally Only	By Injection Only	Orally and Injected Concomitantly	Currently Receiving Penicillin	No Penicillin, No Reaction	Reactions to Other Drugs	Total No. Tests Performed
Procaine penicillin	6	2	0	0	0	2	0	0	8
Bicillin	0	0	0	0	0	0	0	0	2
Sodium penicillin	12	3	1	1	1	0	0	0	12
Potassium penicillin	30	8	1	6	1	0	0	0	8
Procaine + sodium	40	10	1	6	0	0	3	0	....
Procaine + bicillin	0	0	0	0	0	0	0	0	....
Sodium + bicillin	3	1	0	1	0	0	0	0	....
Procaine + bicillin + sodium	3	1	0	1	0	0	0	0	....
Tetanus antitoxin	3	1	....	....	....	....	....	1	1
Chloramphenicol	3	1	....	....	....	....	....	1	1
Total	100	27	3	15	2	2	3	2	32

the result of poor technique. To demonstrate the adequacy of the technique, guinea pig serum (antigen) and rabbit anti-guinea pig serum (antibody) were employed. The result of this test was clearly positive and duplicated results obtained by other workers in the laboratory from which the specimens of serum were obtained. Our experience was not unusual. Mendes,[22] in a study of six patients reputed to have died of penicillin reactions, was not able to find precipitins by the double diffusion technique. Previous investigators were not able to show precipitins in persons who had had reactions to pure penicillin.* Rostenberg and Welch,[37] using a conjugate of crystalline penicillin and human plasma, were unable to show precipitins. Muelling[26] suggested that the penicillin antigen be prepared and allowed to stand in the light for several days, and that no preservative be added to the agar. These directions were followed in repeating the tests with five specimens of serum that had been tested previously, and results were again negative.

The double diffusion plate used in this study might be unsuitable for revealing the presence of precipitin. However, Muelling's technique and the double diffusion technique are the same in theory and essentially the same in practice.[48] They are equally sensitive and equally applicable in similar situations.

Callaway[4] and Welch and co-workers,[44] using a standard fluid technique for demonstrating precipitins, found a faint precipitate at the junction of serum and penicillin in subjects with penicillin reaction and also in one control. They interpreted these reactions as inadequate to prove the presence of precipitin. A similar reaction may have occurred to produce false positives in Muelling's series, but such reactions clearly did not occur in our tests. They may well have been due to the Liesegang phenomenon, thought to be a nonspecific precipitation related to reactant and precipitate concentrations.[42]

It is possible that treatment received by the patient interfered in some way with the precipitin test. If the reaction were dependent on precipitins, however, one would expect to find them afterward, since drug reactions tend to recur on subsequent exposure, regardless of therapy for a previous reaction.

The passive cutaneous anaphylaxis test[30,31] was utilized by us in several studies to determine if pre-

*References 7, 19, 21, 26, 37, 44.

cipitins could be demonstrated by this more sensitive method. In the initial tests, precipitins were not demonstrated.

The infrequent presence of reagin (demonstrated by negative results of direct and passive transfer tests referred to above) seems to eliminate reagin as the cause of drug reactions. It appears that reagin occasionally accompanies reactions but is not the cause of the reaction.

Mechanisms other than these antigen-antibody systems may be involved. Ackroyd,[1] in a classic study, clearly demonstrated antibodies against platelets in a drug reaction to Sedormid® (allyl-isopropyl-acetylcarbamide). Quinine has been similarly suspect, but the mechanism of its action has not been proven.[6]

Enzyme defects are known to mediate some drug reactions. It has been shown, for example, that primaquine sensitivity is a result of a relative lack of glucose-6-phosphate dehydrogenase in the red blood cells of sensitive patients, and this lack is determined by a sex-linked gene of intermediate dominance. Naphthalene and nitrofurantoin (Furadantin®) cause a hemolytic anemia that is associated with a sulfhydryl defect in the red cell. These and other reports of genetically and chemically determined drug reactions are summarized by Motulsky.[23] It is possible, therefore, that penicillin reactions may be mediated in part by some similar mechanism although there is no definitive evidence to support the conjecture.

Since our work was completed, several studies have appeared using the red-cell agglutination technique to demonstrate circulating antibody in the serum of patients allergic to penicillin. Ley[17] reported such tests first in 1958. Bird and co-workers[3] in 1960 have repeated the survey using serum from patients allergic to penicillin, from patients receiving penicillin but not allergic to it, and from patients neither allergic nor receiving penicillin. They noted positive reactions occasionally and from each of the groups, and concluded along with Ley that this was not a useful method of determining penicillin sensitivity and that the mechanism of the penicillin reaction is not yet explained. Vaughan and Harris in 1960[43] reported a smaller number of patients with a larger number of positive tests. Most recently, Heggie[13] reported studies showing that about 30 per cent of 62 patients with penicillin reactions had positive hemagglutination tests, while about 8 per cent of patients without reactions showed positive tests. He concluded this is a useful test, and further work is indicated. In view of these divergent results and opinions, final comment must await future investigation.

300 Homer Ave., Palo Alto.

REFERENCES

1. Ackroyd, J. F.: The role of Sedormid in the immunological reaction that results in platelet lysis in Sedormid purpura, Clin. Sci., 13:409, 1954.

2. Alexander, H. L.: Reactions with Drug Therapy, p. 99, W. B. Saunders Co., Philadelphia, 1955.

3. Bird, G. W. G.: Penicillin antibody, J. Clin. Path., 13:51, 1960.

4. Callaway, J. L., and Barefoot, S. W.: Immunological studies on patients developing urticaria associated with penicillin therapy, J. Invest. Derm., 7:285, 1946.

5. Collins-Williams, C., and Vincent, J.: Sensitivity reactions to penicillin in children, Ann. All., 11:454, 1953.

6. Creger, W. P., and Houseworth, J. H.: Erythrophagocytosis and thrombocytopathy occurring during the course of a Henoch-Schonlein syndrome due to quinine, Am. J. Med., 17:423, 1954.

7. Criep, L. H.: Allergy to penicillin, J.A.M.A., 126:429, 1944.

8. Doniach, D., and Roitt, I. M.: Auto-immunity in Hashimoto's disease and its implications, J. Clin. Endocrin., 17: 1293, 1957.

9. Feinberg, S. M.: Drug allergy—Some clinical and immunological aspects, Ann. All., 10:260, 1952.

10. Glaser, J. G.: Allergy in Childhood, p. 321, Chas. C. Thomas, Springfield, Ill., 1956.

11. Halbert, S. P., Swick, L., and Sonn, C.: The use of precipitin analysis in agar for the study of human streptococcal infections, J. Exp. Med., 101:539, 1957.

12. Harris, M. C., and Shure, N.: Practical Allergy, p. 202, F. A. Davis Co., Philadelphia (no date).

13. Heggie, A. D.: Incidence of circulating antibody to penicillin in penicillin hypersensitivity reactions, N.E.J.M., 262:1160, 1960.

14. Heiner, D. C.: Diagnosis of histoplasmosis using precipitin reactions in agar gel, Ped., 22:616, 1958.

15. Hirsh, H. L., and Putnam, L. E.: Penicillin Antibiotics Monograph No. 9, p. 21, Medical Encyclopedia Inc., New York, 1958.

16. Leftwich, W. B.: An intradermal test for the recognition of hypersensitivity to the sulfonamide drugs, Bull. J. Hop. Hosp., LXXIV No. 1, p. 26, 1944.

17. Ley, A. B., Harris, J. P., Brinkley, M., Liles, B., Jack, J. A., and Cahan, A.: Circulating antibody directed against penicillin, Science, 127:1118, 1957.

18. Lowell, F. C.: Allergic reactions to sulfonamide and antibiotic drugs, Ann. Int. Med., 43:333, 1955.

19. Mallin, M. S., and Cuellar, O.: Immunological studies with sera from penicillin-allergic patients, Ann. All., 7:1, 1949.

20. Matheson, A., and Elegant, L.: Penicillin reactions in children, J. All., 26:415, 1955.

21. Mayer, P. S., Mosko, M. M., Schutz, P. J., Osterman, F. A., Steen, L. H., and Baker, L. A.: Penicillin anaphylaxis, J.A.M.A., 151:351, 1953.

22. Mendes, E., Rodrigues, A., Fernandes, A., Cintra, A. U., and Lacaz, C. S.: Allergic study of seven cases of sudden death ascribed to penicillin, Acta. Allergologica XIV, p. 445, 1959.

23. Motulsky, A. G.: Drug reactions, enzymes, and biochemical genetics, J.A.M.A., 165:835, 1957.

24. Muelling, R. J., Jr., Beven, T., Samson, R. F., Jenevein, E. P., and Guillory, J. D.: Studies in human sensitivity to antibiotics, Am. J. Clin. Path., 29:503, 1958.

25. Muelling, R. J., Jr., Samson, R. F., and Beven, T.: The precipitin test in elucidating the cause of death, Am. J. Clin. Path., 28:489, 1957.

26. Muelling, R. J., Jr.: Personal communication, Nov. 1959.

27. Olsen, C. R.: Penicillin allergy—Incidence in young adults and a review of the subject, Stanford Med. Bull., 16:238, 1958.

28. Ouchterlony, O.: Antigen-antibody reactions in gels IV—Types of reactions in coordinated systems of diffusion, Acta. Path. et Microbiol. Scand., 32:231, 1953.

29. Oudin, J.: Specific Precipitation in Gels and Its Application to Immunochemical Analysis. Methods in Medical Research, Vol. 5, p. 360, Year Book Publishers, Inc., Chicago, Ill., 1952.

30. Ovary, Z.: Quantitative studies in passive cutaneous anaphylaxis of the guinea pig, Int. Arch. All., 3:162, 1952.

31. Ovary, Z., and Bier, O. G.: Quantitative studies on passive cutaneous anaphylaxis in the guinea pig and its relationship to the Arthus phenomenon, J. Imm., 71:6, 1953.

32. Parlett, R. C., and Youmans, G. P.: Antigenic relationships between ninety-eight strains of Mycobacteria using gel-diffusion precipitation, Am. Rev. Tub., 77:450, 1958.

33. Peck, S. M., Siegal, S., Glick, A. W., Kurtin, A., and Bergamini, R.: Clinical problems in penicillin sensitivity, J.A.M.A., 138:631, 1948.

34. Rabinowitz, H., and Halpern, S. R.: Drug allergy in pediatric practice, J. Ped., 56:75, 1960.

35. Raffel, S.: Immunity, Hypersensitivity, and Serology, p. 265, Appleton-Century-Crofts, Inc., New York, 1953.

36. Rajka, G., Jr., and Vincze, E.: Penicillin combined with gamma globulin as a diagnostic agent in urticaria of the serum sickness type due to penicillin, Ann. All., 16:291, 1958.

37. Rostenberg, A., Jr., and Welch, H.: A study of the types of hypersensitivity induced by penicillin, Am. J. Med. Sc., 210:158, 1945.

38. Sheldon, J. M., Lovell, R. B., and Mathews, K. P.: A Manual of Clinical Allergy, p. 128, W. B. Saunders, Philadelphia, 1953.

39. Sherman, W. B., and Kessler, W. R.: Allergy in Pediatric Practice, p. 253, C. V. Mosby Co., St. Louis, 1957.

40. Siegal, S.: Allergic reactions to antibiotics, N. Y. State J. Med., 55:2303, 1955.

41. Stier, R. A., and Stier, R. F. E.: The results of desensitization in allergy to insect stings, G. P., 19:103, 1959.

42. van Oss, C. J., and Hirsh-Ayalon, P.: An explanation of the Liesegang phenomenon, Science, 129:1365, 1959.

43. Vaughan, J., and Harris, J.: An investigation of hemagglutinating antibody to penicillin, Abst. Scien. Meet. Amer. Acad. Allergy, 1960, p. 30.

44. Welch, H., and Rostenberg, A., Jr.: Hypersensitivity of the tuberculin type to crystalline penicillin sodium, J.A.M.A., 126:10, 1944.

45. Wodehouse, R. P.: Analysis and stabilization of house dust by gel diffusion, Ann. All., 12:363, 1954.

46. Wodehouse, R. P.: Antigenic analysis by gel diffusion, Ann. All., 15:527, 1957.

47. Wodehouse, R. P.: Antigenic analysis and standardization of Trichinella extract by gel diffusion, Ann. All., 14:121, 1956.

48. Wodehouse, R. P.: Gel diffusion, Ann. All., 14:96, 1956.